

GLYCOENGINEERING OF *ASPERGILLUS NIDULANS* USING AN OCH1-GNTI FUSION PROTEIN

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Abstract

Glycoproteins are an integral type of treatment to modern medicine.

Glycoproteins are proteins which are adorned with sugar chains known as glycans.

Asparagine-linked glycans, also called N-glycans, are of particular importance. They are involved in many cellular processes such as cell signaling, protein folding, and secretion of products. While the beginning of the N-glycosylation pathway is mostly the same across eukaryotic organisms, the glycosylation pathways diverge in later steps, causing foreign N-glycans to be immunogenic to humans. This limits the possible production hosts for such glycoproteins used in medicine to hosts which produce similar enough glycans to humans that they will not cause immune rejection. In the industry today, most glycoproteins are produced by mammalian cell lines such as Chinese hamster ovary (CHO) cells. These cell lines produce glycoproteins containing glycans very similar to humans, however, they are very expensive to culture. Filamentous fungi such as *Aspergillus nidulans* produce and secrete glycoproteins at a much higher rate and grow in cheaper media, however, the glycans they produce are immunogenic to humans.

In order to obtain more cost-effective glycoprotein production, *A. nidulans* can be engineered to produce human-like glycans. To achieve this end, this study uses an *A. nidulans* strain which has been engineered to produce glycans with fewer mannose residues and inserts an och1-GnTI fusion protein. The och1 section of this fusion protein is meant to direct the protein to the Golgi apparatus membrane, where it can then add N-acetylglucosamine (GlcNAc) to the glycans. This is a necessary step in the process to achieve humanized glycosylation in *A. nidulans*. Expression and activity of this fusion protein is analyzed using qRTPCR, Western, and lectin blotting. The qRTPCR and

Western blot both show expression of GnTI, and the lectin blot indicates additional GlcNAcylation in the transformant strains compared to the parent strain. Both transformant strains produced in this study appear to express GnTI and actively GlcNAcylate glycoproteins.

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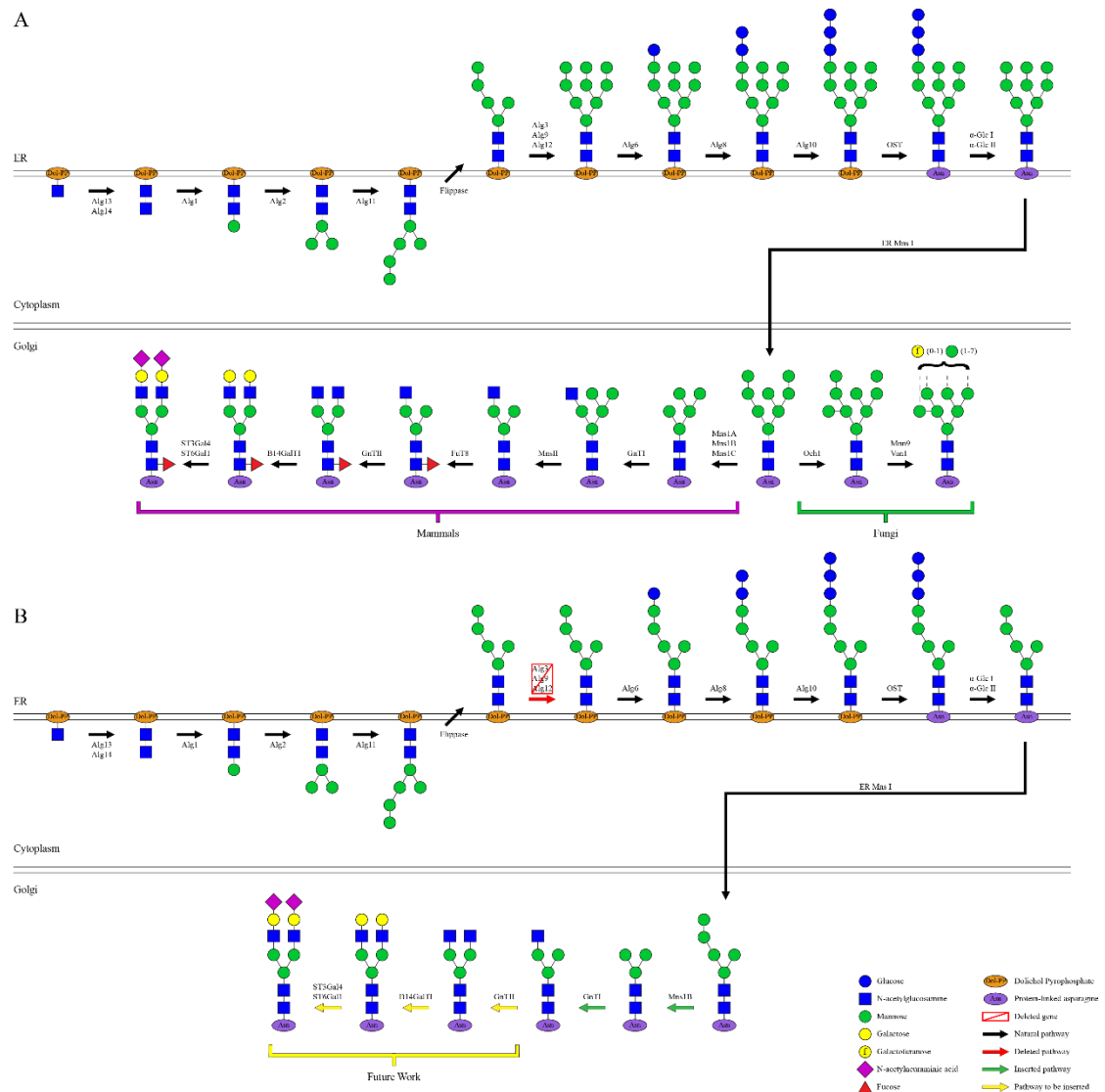
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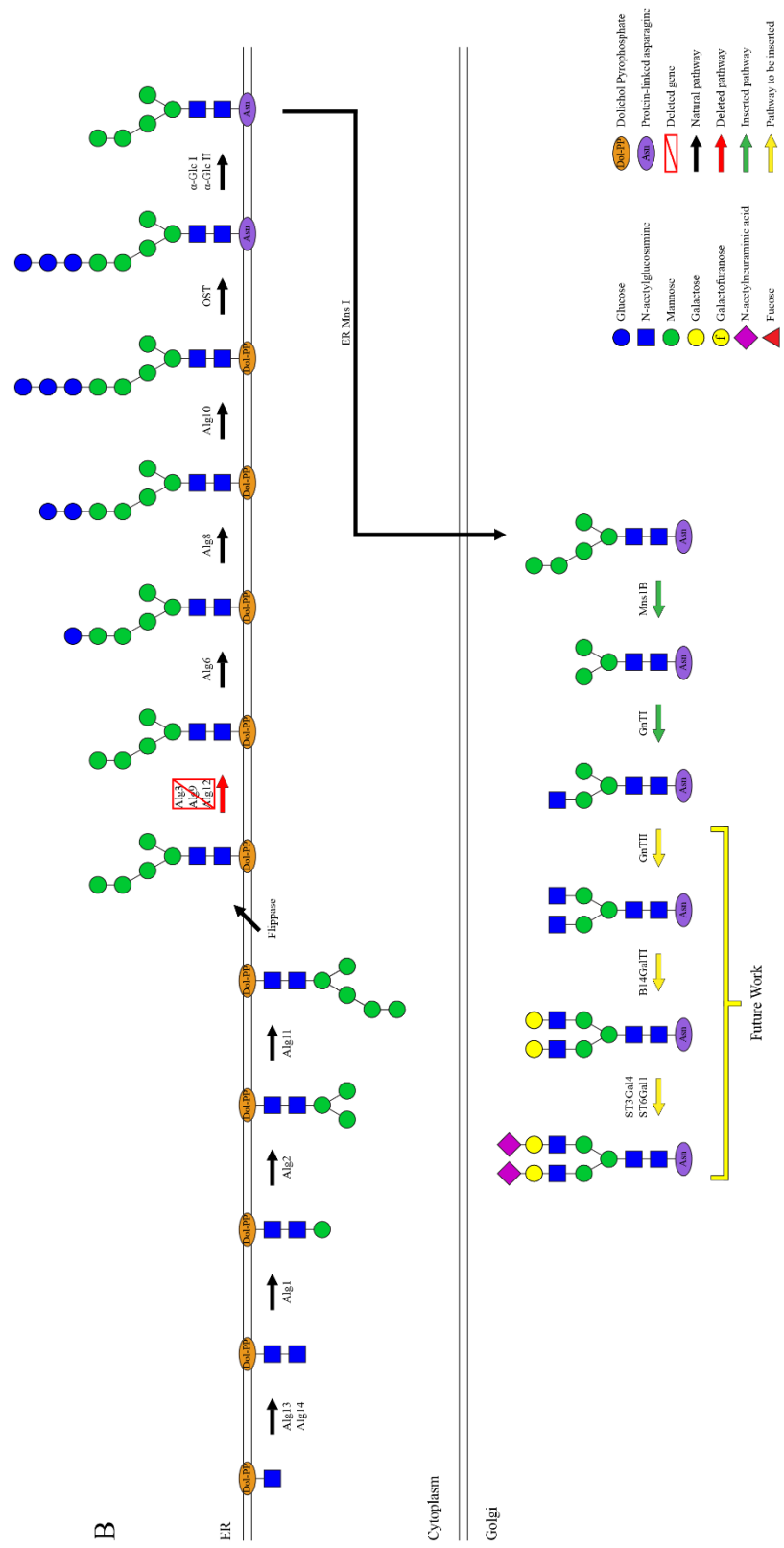
Chapter 1. Introduction

In protein therapeutics, N-glycans play an important role in the efficacy of treatments. Most eukaryotes attach sugars to proteins at NXS/T amino acid motifs, where X is any amino acid except proline, in a process known as N-glycosylation [1]. These glycans have widespread effects in a cell, being involved in processes such as protein folding, cell signaling, and product secretion [2]. The glycosylation pathways in most eukaryotes are identical in the earlier steps, however, species of different kingdoms tend to utilize different patterns of monomers in the construction of glycans in the process' later phases [3]. The result is that the glycan structures can vary greatly between organisms. As the result of this variation, the human immune system detects and quickly disposes of foreign glycoproteins [4, 5]. Because of this, glycoprotein therapeutics are usually produced in closely related mammalian cells, most commonly Chinese hamster ovary (CHO) cells [6].

While CHO cells are useful for their ability to produce similar glycans to those found in humans, they are outclassed in terms of protein production rate by filamentous fungi . After years of improving culturing methods for productivity, CHO cells have reportedly reach a product yield of 4.7 g/L, while yields exceeding 20 g/L have been reached using the filamentous fungus *Aspergillus niger* [7, 8]. Fungi naturally secrete proteins as a method of breaking down extracellular carbon sources for consumption. Because of this natural necessity, fungi have evolved a more well-tuned secretion system, giving them a higher protein secretion rate. Additionally, production of medically relevant proteins in fungi is advantageous because fungal growth media is cheaper and characterized as opposed to mammalian growth media, which is expensive and

proprietary, so its components are unknown. Fungal cultures are also not susceptible to viral contamination, which can be a major production cost in mammalian production systems [9]. Between all of these advantages, fungal expression systems represent a promising alternative to mammalian systems for producing therapeutic proteins, as they would allow for quicker and more cost effective production of these proteins.





The largest obstacle in utilizing fungi, however, is that their glycosylation pathway is very different from that of mammals. While mammalian glycosylation pathways often result in what are known as complex glycans, fungal pathways result in high-mannose glycans, as seen in Figure 1 [10]. The complex glycans of mammals are characterized by their use of several different sugar monomers, notably N-acetylglucosamine (GlcNAc), galactose, and N-acetylneuraminic acid, commonly referred to as sialic acid. In contrast, the high-mannose glycans of filamentous fungi are characterized by their large, branching structures consisting almost entirely of mannoses [11]. These high-mannose glycans are immunogenic in humans. Therefore, in order to utilize fungi to their full potential as production hosts, their glycosylation pathways must be engineered to result in glycans resembling those produced by a human pathway.

In order for fungi to be used to produce nonimmunogenic glycoproteins, they should produce a glycan resembling that shown at the end of the mammalian glycosylation pathway in Figure 1. While the human glycome consists of several glycan structures of a wide variety, this particular glycan serves as a good model due to its sialic acid caps and requiring less transformation steps to be achieved [12]. Sialic acid is of particular importance because it plays a key role in extending the circulatory lifetime of the glycoprotein [13]. Glycoproteins with glycans containing terminal galactose or GlcNAc residues are cleared from the circulatory system by the asialoglycoprotein-receptor (ASGP-R), expressed by liver parenchymal cells. Glycoproteins with terminal sialic acid residues, however, are not cleared by ASGP-R, increasing their circulatory lifetime [14]. As a consequence of increased circulatory lifetime, glycoprotein therapeutics containing terminal sialic acids on their glycans also have increased efficacy

[15]. To achieve terminally sialylated glycans from a fungal expression host, the mannosylation which is natural to fungi must be reduced, and mammalian glycosylation pathways must then be introduced into the fungi.

In this study, an engineered strain of *Aspergillus nidulans* has been used, known as *A. nidulans* 2161 [16]. This strain has had the *nkuA*, *alg3*, *alg9*, and *alg12* genes deleted, as well as an α -mannosidase, glucosidase II, Mns1B, and β -glucosidase overexpressed. *Alg3*, *9*, and *12* function as mannosyltransferases in the endoplasmic reticulum (ER). By targeting the enzymes acting early on in the glycosylation pathway, the resulting glycan can more easily be reduced to Man3GlcNAc2, a template structure which is common to mammals and fungi, which will then be built off of by the insertion of mammalian glycosylation genes. The added α -mannosidase also serves to reduce the extent of mannosylation in order to achieve this starting template by cleaving the α -1,2 mannoses attached to the α -1,3 mannose branch [17]. The glucosidase II is an enzyme that cleaves glucose caps which, when overexpressed, was shown to allow the fungi to produce a higher proportion glycans consisting of five or less mannoses [16]. The overexpressed β -glucosidase is secreted and serves as a model protein to study the effects of these genetic modifications on glycosylation.

The *nkuA* gene, which has been deleted in this strain, is a gene involved in the non-homologous end joining (NHEJ) DNA repair pathway [18]. This is one of two DNA repair pathways utilized by fungi, the other being homologous recombination. Homologous recombination is often used as a method of targeted gene insertion or deletion in fungi [19]. It utilizes large lengths of homologous regions of DNA upstream and downstream of both the target genomic site and the insert, and lines up these

homologous regions to “repair” the damaged genomic DNA using the insert DNA as a template, resulting in the insertion or deletion of a gene of interest. NHEJ naturally opposes this process, as it is an alternative DNA repair mechanism which would not result in the usage of transformed DNA as a template, therefore not resulting in any genomic sequence changes. By deleting *nkuA*, NHEJ becomes drastically less functional, requiring that homologous recombination be used for DNA repair, thus increasing the success rate of targeted gene insertion or deletion.

This study aims to build off the Man3-producing strains engineered by Anyaogu *et. al* [16]. To accomplish this, the gene encoding a human GlcNAc transferase, GnTI, has been introduced into *A. nidulans* 2161. GnTI is a membrane protein which must be localized to the Golgi apparatus in order to properly modify glycans, as this is where glycoproteins are sent for late-stage glycan modification and subsequent secretion [20]. Proteins that are designated to particular subcellular locations require signal peptides to be directed to their target destination correctly. These signal peptides can vary between species. Additionally, the mammalian transmembrane domain may be less functional in fungi due to differing membrane compositions between the species. Therefore, it is important that the signal peptide and transmembrane domains of the mammalian GnTI be replaced with an alternative that will function in *A. nidulans*. Similar studies have used a combinatorial library in order to target GnTI to the fungal Golgi apparatus in the past; however, in this study, the signal peptide and transmembrane domain of the putative fungal mannosylation gene, *och1*, were used to replace the human signal peptide and transmembrane domain, creating a fusion protein, f-GnTI [21].

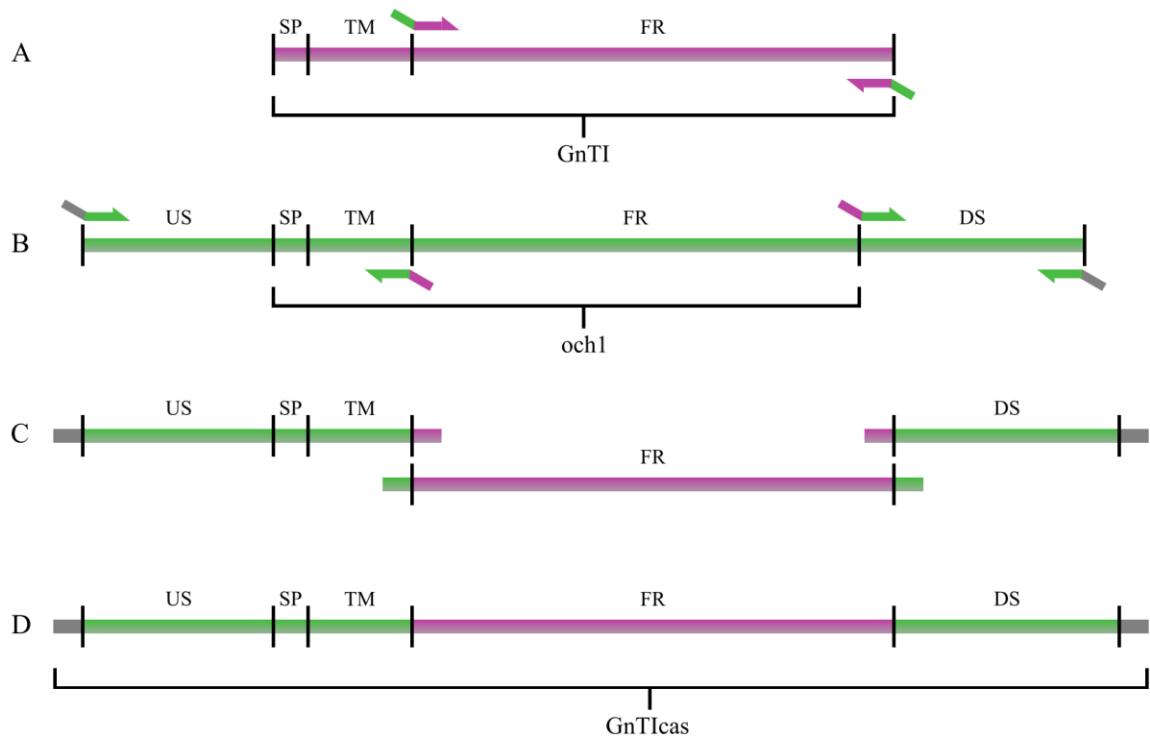


Figure 2. Gene construct. A) GnTI gene, consisting of a signal peptide (SP), transmembrane domain (TM), and functional region (FR). Primer annealing sites and overhangs are represented by arrows. B) Och1 gene, including the upstream (US) and downstream (DS) regions. Grey primer overhangs represent restriction sites, with NotI on the US side and XbaI on the DS side. C) Initial PCR products shown overlapping at sites where annealing occurs during fusion PCR. D) Final gene construct. Segment lengths are not to scale.

In order for f-GnTI to be targetedly inserted into the fungal genome, a three-part gene construct was built. This construct consists of the f-GnTI gene itself and an upstream and downstream homologous region, which are homologous to sections in the fungal genome and flank the GnTI gene on each end, targeting the gene to the genomic location at which the homologous regions align. The *och1* putative gene encodes a mannosyltransferase which acts early on in the glycosylation pathway in the Golgi [11]. Furthermore, *och1* adds a mannose residue as an additional branch on the glycan, providing a mannose substrate for subsequent Golgi-localized mannosyltransferases to act on without needing to compete for substrate with GnTI, which has specificity for the

α -1,3 mannose branch [3]. Ultimately, och1 activity would result in undesirable mannosylation of the glycans. By flanking the GnTI insert with the upstream and downstream sequences of och1, homologous recombination can be used to simultaneously delete the unwanted och1 and replace it with GnTI. Additionally, by utilizing only the functional region of the GnTI gene without its native signal peptide and transmembrane domain, these regions can be replaced with the fungal equivalents that are native to och1 by leaving the och1 signal peptide and transmembrane domains as the 3' end of the upstream homologous region used for targeting GnTI to the och1 gene's location in the genome. This method also allows for any native promoters and enhancers for och1 to be used to express GnTI instead.

Chapter 2. Background

2.1. 5-Fluoroorotic Acid Counterselection

In this study, 5-fluoroorotic acid (5-FOA) counterselection was utilized to reuse the pyrG selection marker for transforming the fungi. PyrG is an auxotrophic selection marker which is involved in the synthesis of uracil from orotidine 5-phosphate [22]. Strains which are pyrG deficient must be supplemented with uridine and uracil in order to grow. To utilize this, the pEXpyr plasmid used in this study contains a pyrG selection marker, which restores the fungi's ability to synthesize uracil, allowing them to grow on selective plates which do not contain uridine or uracil. The pyrG gene is flanked by mate1 sequences, which are direct repeats [23]. Fungi will randomly undergo a recombination event in which the sequence between these mate1 sequences will be excised from the genome. 5-FOA is a fluorinated molecular mimic of orotidine 5-phosphate, which, when converted to 5-fluorouracil by the activity of pyrG, becomes toxic to the cell and kills it. This allows for the selection of those colonies which have undergone the random recombination event and thus have removed the pyrG selection marker, allowing it to be reused in subsequent transformations [24]. The gene that was transformed into the fungi is not lost with the pyrG selection marker because it is not in between the mate1 sequences.

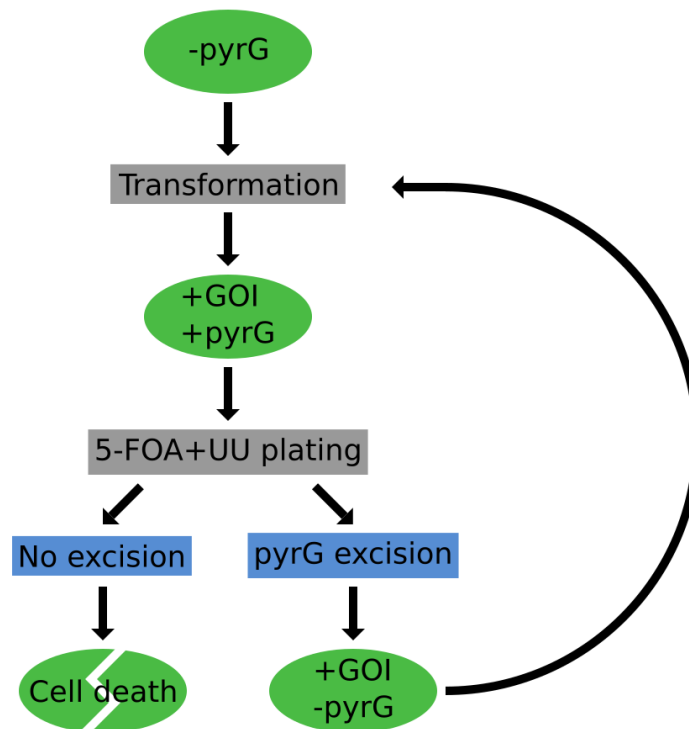


Figure 3. 5-FOA counterselection process. Fungal genotypes are shown in green ovals. Gene of interest is represented by “GOI.”

2.2. qRTPCR

The expression of GnTI at the mRNA level was measured using quantitative reverse transcriptase PCR (qRTPCR). The basis of this method lies in the use of an intercalating dye which increases the fluorescence of the solution as the amount of DNA increases. By using a thermocycler that can measure fluorescence, comparisons of DNA concentration can be made in real time as the PCR process is carried out. The template DNA for each sample is the cDNA made by reverse transcribing the total RNA of a culture. Because PCR doubles the amount of DNA with each cycle, the amount of DNA present in the sample at any given time, and thus the fluorescence of the sample, directly correlates with the initial amount of cDNA template for the target region. A threshold fluorescence value is computationally decided, which is the lowest fluorescence value

that is statistically significantly different from the baseline fluorescence reading. The cycle at which each sample reaches this threshold value, C_T , is recorded. Due to the exponential nature of PCR, it can be concluded that the ratio of starting amount of cDNA for a target gene in one sample compared to another is equal to $R_E = 2^{CE_{T2} - CE_{T1}}$, where R_E is the ratio of initial cDNA of the experimental gene in sample 1 compared to sample 2, CE_{T1} is the cycle at which sample 1's fluorescence reached the threshold value, and CE_{T2} is the cycle at which sample 2's fluorescence reached the threshold value. In order to control for difference between the initial amount of cDNA loaded into each sample, a housekeeping gene is usually used. A housekeeping gene can be any gene which is expressed at an equal amount in each experimental and control sample. Actin and histone genes are a few examples of commonly used housekeeping genes. To correct for differential sample loading, a ratio of expression of the housekeeping gene must be found using $R_H = 2^{CH_{T2} - CH_{T1}}$, where R_H is the ratio of initial cDNA for the housekeeping gene between samples 1 and 2, and CH_{T1} and CH_{T2} are the cycles at which samples 1 and 2 reached the fluorescence threshold, respectively. The ratio of expression of the experimental gene can then be found by using $R = R_E / R_H$.

2.3. Western and Lectin Blotting

Western and Lectin blots are methods for visualizing the presence or absence of target proteins. They function by loading a solution containing several types of proteins, often cell lysate or media, into an SDS-PAGE gel and running an electrical current through the gel to separate the proteins by size. These proteins are then transferred to a membrane which binds proteins. In Western blotting, an antibody for a protein of interest is used, which will specifically bind to the protein of interest if it is present. This

antibody is referred to as the primary antibody. A secondary antibody is then used which has specificity for the constant region of the primary antibody and is attached to a conjugate which enables visualization of the proteins. Lectin blots, rather than utilizing antibodies with protein specificity, rely on lectins. Lectins are a class of protein which have specific binding for monosaccharides which can commonly be found on glycans. Lectins can be conjugated to a visualization molecule directly or to biotin, which allows any visible molecule conjugated to streptavidin to be used to visualize the lectin, and by extension any glycoprotein which the lectin has bound to.

Chapter 3. Materials and Methods

3.1. Strains and Media

The parent strain used in this study was *Aspergillus nidulans* 2161 [16]. From this strain, *A. nidulans* 3162 and 3168 were created by transforming *A. nidulans* 2161 with the human MGAT1 gene, which was randomly inserted into the genome, yielding these two transformant strains. All strains were grown at 28 °C for agar or liquid media, and strains grown in liquid media were grown in a shaking incubator at 250 RPM unless otherwise stated. The media and agar recipes used in this study are shown in Table 1 [25].

MAGV	
Component	Concentration
Malt extract	20 g/L
Peptone	2 g/L
Glucose	20 g/L
Vitamin mix	1X
Hutner's Solution	1X
YGV	
Yeast extract	5 g/L
Glucose	20 g/L
Vitamin mix	1X
Hutner's Solution	1X
Minimal Media + Sorbitol	
D-Sorbitol	1.2 M
Glucose	20 g/L
Vitamin mix	1X
Hutner's Solution	1X
Ammonium Chloride	5 g/L
Minimal Media	
Glucose	10 g/L
Magnesium Sulfate	
Nitrate Salts	1X
Vitamin mix	1X
Hutner's Solution	1X
Vitamin mix (1000X)	
Biotin	100 mg/L
Pyridoxin	100 mg/L
Thiamine	100 mg/L
Riboflavin	100 mg/L
P-aminobenzoic acid	100 mg/L
Nicotinic acid	100 mg/L

Hutner's Solution (1000X)	
Component	Concentration
ZnSO ₄ • 7H ₂ O	22 g/L
H ₃ BO ₃	11 g/L
MnCl ₂ • 4H ₂ O	5 g/L
FeSO ₄ • 7H ₂ O	5 g/L
CoCl ₂ • 6H ₂ O	1.6 g/L
CuSO ₄ • 5H ₂ O	1.6 g/L
(NH ₄) ₆ Mo ₇ O ₂₄ • 4H ₂ O	1.1 g/L
EDTA	50 g/L

Table 1. Media compositions

Media requiring uracil and uridine (UU) supplements have 1.12 g/L and 1.22 g/L of uracil and uridine, respectively, in addition to the media's normal components as listed above. Agar-containing media was made using the listed recipes above with the addition of 2% w/v agarose. MAGV+UU+5-FOA agar was made using 1.3 mg/mL 5-FOA [26].

3.2. Strain Banking

Upon receiving *A. nidulans* 2161 or upon creating a new strain, strains were banked by first growing them in liquid MAGV media, supplemented with uridine and uracil if necessary. After 2-3 days of growth, 300 µL of media were plated onto each of 3 MAGV agar plates, supplemented with uridine and uracil if necessary, and allowed to grow for 3 days. 8 mL of PBS was then pipetted onto each plate, and the spores scraped off using a scraper. The PBS and spore solutions were combined and then vortexed at high speed for 60 seconds. The solution was then pushed through a sterile 60-mL syringe with sterile glass wool in the bottom, removing the mycelia from the spore solution. Spores were then counted and diluted using PBS to 1x10⁷ spores/mL in a solution with

20% glycerol. The spore solutions were then split into 1-mL aliquots and frozen at -80 °C.

3.3. 5-FOA Counterselection

After banking *A. nidulans* 2161, it was first grown on MAGV+UU+5-FOA plates. After growing for 7 days at 28 °C, colonies were then picked using a sterile pipette tip and grown in MAGV+UU liquid media for banking.

3.4. Gene and Plasmid Construction

The plasmid backbone used in this study was pEXpyr. The och1 upstream and downstream regions were first PCRRed from *A. nidulans* 2161 genomic DNA using primers och1_US_F and och1_US_R for the upstream region and och1_DS_F and och1_DS_R for the downstream region. GnTI with a truncated signal peptide and transmembrane domain (GnTI-dTM) was PCRRed from *E. coli* lysate containing the human gene for GnTI using primers GnTI_dTM_F and GnTI_R. *E. coli* containing GnTI was ordered from DNASU. PCR products were then gel extracted using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol with modifications to increase yield. Modifications include running the initial sample through the column three times and allowing the PB buffer to evaporate for 5 minutes before adding nuclease free water to the column, which was used in place of elution buffer. The nuclease free water was also allowed to sit on the column for 5 minutes before being centrifuged through. 1 µL of each of the och1 upstream region and GnTI-dTM were then used as template in a subsequent fusion PCR reaction. The product was then gel extracted, and 1 µL of purified product was then used with 1 µL of the och1 downstream DNA in another PCR reaction. All PCR reactions were conducted using Phusion HF polymerase (New

England Biolabs) according to manufacturer's instructions. The completed and purified construct and pEXpyr were then digested using NotI and XbaI restriction enzymes (New England Biolabs). The insert and plasmid were then cleaned up using a QIAquick PCR Purification Kit (Qiagen), used according to the manufacturer's protocol with the same modifications listed for the gel extraction protocol, and were ligated at room temperature for 15 minutes in a 5:1 insert to vector ratio using T4 DNA ligase (New England Biolabs), creating the plasmid pEXpyr-GnTicas. 2 μ L of the ligation reaction were then used in a transformation into NEB 5-alpha competent *E. coli* (New England Biolabs) according to the manufacturer's protocol.

Primers	
Name	Sequence (5' - 3')
och1_US_F	<u>AAGCGGCCG</u> <u>CCTTTATCTAATCAGAGCCGAGCAG</u>
och1_US_R	<u>GGCGCGTAGAATGCGACGATCGAAGGA</u>
och1_DS_F	<u>CCTAGCTGGAATTAGGAAGATCATCTTTT</u> <u>AGTGCCCAAGAT</u>
och1_DS_R	<u>AATCTAGAACATCGTTACTGTGGGCGAA</u>
GnTI_dTM_F	<u>GTCGCATTCTACGCGCCCAGCACCT</u>
GnTI_R	<u>AAGATGATCTTCCTAATTCAGCTAGGATCATAGCCC</u>
och1_US800_F	<u>AAAAAACCTCAGCGATACAACATGACAACATCGATCC</u>
och1_DS800_R	<u>AAAAAATCTAGACTCAGAGGCATATACAGGTTGTC</u>
GnTI_mRNA_F	<u>CCAGCTGGACCTGTCTTACC</u>
GnTI_mRNA_R	<u>CGGAACTGGAAGGTGACAAT</u>
His2B_mRNA_F	<u>CACCCGGACACTGGTATCTC</u>
His2B_mRNA_R	<u>GAATACTTCGTAACGGCCTTGG</u>

Table 2. Primer sequences. Underlined regions represent overhangs.

3.5. Transformation

Flasks containing 150 mL of YGV+UU media were first inoculated with 1×10^7 spores of counterscreened *A. nidulans* 2161 and grown at 28°C and 200 RPM for 24 hours. Cultures were then slowly poured through a nylon mesh with regular shaking of the flask to separate the media from the mycelia. The mycelia were then scraped into a 50-mL conical tube using a sterile spatula. The 50-mL conicals were then filled with 20-

30 mL of lysing enzyme solution containing 6 g/L Lallzyme MMX (Lallemand). The tube was then secured on its side and incubated at 30 °C while shaking at 100 RPM for 2 hours. After incubation, the solution was poured through 2 layers of sterile Miracloth in a funnel into a new 50-mL conical tube. The protoplast solution was then centrifuged at 4000 xg and 4°C for 10 minutes. The supernatant was removed and the pellet was then resuspended in 50 mL STC 50. STC 50 is made up of 218.6 g/L D-Sorbitol, 1.48 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1/20 of the total volume of Tris HCl pH 7.5 (1 M). The protoplasts were then centrifuged and resuspended in 50 mL of STC 50 a second time. After the final wash, the protoplasts were centrifuged at 4000 xg and 4°C for 10 minutes, and then resuspended in 1 mL STC 50. 150 μL of this protoplast solution was taken and added to a 15-mL conical tube. 10 μg of pEXpyr plasmid with insert was then added to the 150- μL protoplast solution, and the volume filled up to a total of 200 μL with STC 50. This solution was then incubated at room temperature for 20 minutes. After incubation, 2 mL of 60% PEG-8000 solution was added to the protoplasts, and the solution was incubated for another 15 minutes at room temperature. STC 50 was then added to bring the total volume up to 12 mL, and the tube was inverted gently until the solution was thoroughly mixed. 2 mL of this solution was then plated onto each of 6 plates containing MM+Sorbitol+Agar and allowed to grow for 6 days. Transformant colonies were then picked using a sterile pipette tip and each replated onto a new MM+Sorbitol+Agar plate and allowed to grow for 5 days.

3.6. Transformant Screening

Transformants which showed growth after replating were picked using a sterile pipette tip and grown in 50 mL of MAGV media for 3 days. Some of the media was

taken and plated onto MAGV plates for banking, and the rest was used for genomic DNA extraction using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek) according to the manufacturer's protocol. The extracted genomic DNA was then used as template in two PCR reactions for each transformant using primers och1_US_F and och1_DS_R for the first reaction and och1_US800_F and och1_DS800_R for the second. The PCR products were then run on an electrophoresis gel, and bands corresponding to 2100 bp from the first reaction and 2900 bp from the second reaction were gel extracted and sequenced. Sequencing revealed two colonies which contained the GnTIIcas insert, *A. nidulans* 3162 and 3168, which were then used for further analysis.

3.7. qRTPCR

Strains were first inoculated into 50 mL of YGV media and grown for 24 hours. The contents of the 50-mL culture were then poured into a 2.8-L flask filled with 1 L of YGV. The 1-L culture was then allowed to grow for 2 days. The media was then poured through 2 layers of Miracloth in a funnel. After the entire culture had been run through, remaining media was squeezed out of the mycelia clump. Smaller clumps of mycelia were then broken off and freeze-dried using liquid nitrogen, and then ground using a mortar and pestle while being kept frozen using liquid nitrogen until the mycelia became a fine powder. This lysate was then used in the RNeasy Plus Universal Mini Kit (Qiagen) and RNA was extracted using the manufacturer's protocol.

Extracted RNA was then used in a reverse transcriptase PCR reaction using random primers. The cDNA obtained from the RTPCR was then amplified using primers GnTI_mRNA_F and GnTI_mRNA_R in qPCR reactions using SYBR Select Master Mix

(Applied Biosystems). Additional qPCR reactions of the cDNA templates using primers His2B_mRNA_F and His2B_mRNA_R were carried out in parallel as loading controls.

3.8. Western Blotting

Crude protein extract was obtained by first inoculating 200 mL cultures with 2×10^7 spores and allowing them to grow for 3 days. Mycelia were then extracted and lysed by freeze drying and crushing with a mortar and pestle, as described in section 3.7. Lysate was then dissolved in RIPA buffer by continuous rotation for 1 hour at 4°C and centrifuged to remove cellular debris. Protein concentration of prepared samples was then measured with a BCA assay.

Protein from lysate for each sample was loaded in duplicate onto an SDS-PAGE gel, with 8 µg of protein in one well and 16 µg in a second. The SDS-PAGE gel was run and proteins were then transferred onto a PVDF membrane at 100 V for 75 minutes. Western blot was performed using PBST as a wash buffer and Carbo-Free Blocking Solution (Vector Labs) as a blocking buffer. Goat pAb to B3GNTI (Abcam) was used as the primary antibody to screen for GnTI expression. Membrane was incubated overnight at 4°C with gentle shaking in primary antibody solution containing blocking buffer diluted using PBST to 1:5 blocking buffer to total solution and 0.15 µg/mL of primary antibody. HRP horse anti-goat IgG antibody (Vector Labs) was used as the secondary antibody, which was bound by gently shaking the membrane at room temperature for 2 hours in 1:5 blocking buffer diluted in PBST.

3.9. Lectin Blotting

Protein from lysate for lectin blotting was prepared as described above. Secreted protein was prepared by first growing inoculating 200 mL of minimal media with 2×10^7

spores of *A. nidulans* strains and allowing them to grow in a shaking incubator for 2 days. Mycelia were separated from the media by filtering the media through a miracloth filter in a funnel and squeezing out any additional media from the mycelia clump. The protein concentrations of each sample were measured using a BCA assay, and 6 µg of protein were loaded for each sample into an SDS-PAGE gel. After running the SDS-PAGE gel, proteins were transferred to a PVDF membrane at 100 V for 75 minutes. Membrane washes were performed using PBST. Biotinylated wheat germ agglutinin (WGA) was used to bind terminal GlcNAc residues [27]. The membrane blocked using Carbo-Free blocking solution (Vector Labs). WGA binding was performed by incubating the membrane overnight with gentle shaking at 4°C in PBST with 5 µg/mL WGA. Horseradish peroxidase streptavidin ELISA grade (HRP-streptavidin) (Vector labs) was used for visualization, and was bound to WGA by incubating the membrane for 1 hour with gentle shaking at room temperature in 5 µg/mL HRP-streptavidin in PBST.

3.10. Coomassie Blue Staining

The samples used in the lectin blot described above were loaded into an additional SDS-PAGE gel. After running, the gel was incubated in Coomassie blue overnight with gentle shaking at 4°C for initial staining. The gel was then washed in destaining buffer for 2 hours with gentle shaking at room temperature. The destaining buffer was changed out for fresh destaining buffer and incubated for an additional 2 hours before the buffer was changed out for PBST.

Chapter 4. Results

4.1. Transformant Screening

Transformations were initially attempted by growing *A. nidulans* 2161 in YGV for 12 hours, as was typical for transformations of parent *A. nidulans* strains using this protocol. After this 12 hour period, however, the culture did not appear sufficiently cloudy when using *A. nidulans* 2161, as was normal when using parent strains, likely due to the heavy genetic modification of this strain. Consequently, the initial growing step was increased from 12 to 24 hours. Additionally, fungi were not surviving the transformation protocol when using a 3-hour incubation step in the lysing enzyme solution, which was normal when working with parent *A. nidulans* strains.

Transformation of *A. nidulans* 2161 was successful after reducing this incubation step to 2 hours.

After transformation of *A. nidulans* 2161 with pEXpyr-GnTicas, the genomic DNA of 6 resulting colonies was isolated and used as a template in PCR reactions using primers och1_US_F and och1_DS_R for one amplification and och1_US800_F and och1_DS800_R for a second. Of these, bands corresponding to 2100 bp from the first reaction and 2900 bp from the second were gel extracted and sequenced. Sequencing using och1_US_F and och1_DS_R revealed that *A. nidulans* 3162 and 3168 amplicons both aligned with GnTI, however, sequencing using och1_US800_F and och1_DS800_R returned sequences which did not align with GnTI, but aligned with och1 instead.

Template DNA	Primer	GnTI	och1 US	och1	och1 DS
<i>A. nidulans</i> 3162	och1_US_F	x	x		
	och1_DS_R	x			x
	och1_US800_F		x	x	
	och1_DS800_R			x	x
<i>A. nidulans</i> 3168	och1_US_F	x	x		
	och1_DS_R	x			x
	och1_US800_F		x	x	
	och1_DS800_R			x	x

Table 3. Transformant sequencing results summary. Boxes filled with “x” represent results in which the sequence from genomic DNA aligned with the listed genes.

4.2. qRTPCR

A. nidulans 2161, 3162, and 3168 mRNA was prepared, and cDNA was created from the mRNA templates using RTPCR. The cDNA of each strain was then amplified using qPCR for GnTI and Histone 2B in separate PCR reactions, each run in triplicate, to verify the expression of GnTI at the mRNA level in *A. nidulans* 3162 and 3168.

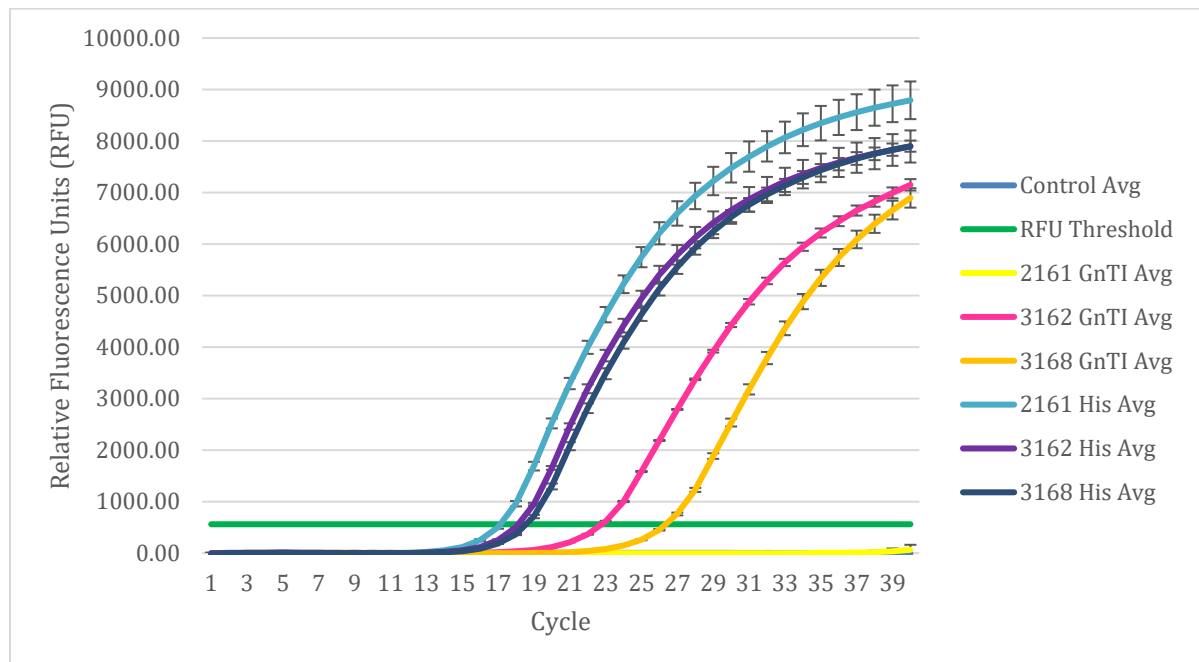


Figure 4. qRTPCR for GnTI. Amount of DNA present in each sample is tracked over the course of the PCR protocol using fluorescence from SYBR Green. Error bars represent standard deviation. “Control Avg” is the average fluorescence from an empty well. “RFU Threshold” is an approximation of the threshold value at which C_T is recorded, shown here for visual demonstration.

Gene	Strain	Replicate	C _T	Average C _T
GnTI	<i>A. nidulans</i> 2161	1	N/A	N/A
		2	N/A	
		3	N/A	
	<i>A. nidulans</i> 3162	1	22.79	22.78
		2	22.73	
		3	22.82	
	<i>A. nidulans</i> 3168	1	26.32	26.35
		2	26.31	
		3	26.42	
His2B	<i>A. nidulans</i> 2161	1	17.07	17.13
		2	17.12	
		3	17.21	
	<i>A. nidulans</i> 3162	1	18.14	18.13
		2	18.09	
		3	18.16	
	<i>A. nidulans</i> 3168	1	18.65	18.57
		2	18.54	
		3	18.50	

Table 4. qRTPCR C_T values.

It can be seen in Figure 4 that the fluorescence value does not rise when amplifying *A. nidulans* 2161 cDNA for GnTI. Additionally, fluorescence rises and a C_T value is returned for *A. nidulans* 2161 Histone 2B, indicating that cDNA from the strain is present, but it does not express f-GnTI nor do the primers amplify any off-target regions. Using the equations from 2.2, we find the ratio of mRNA for f-GnTI present in *A. nidulans* 3162 when compared to *A. nidulans* 3168 to be

$$R_E = 2^{C_{ET2}-C_{ET1}} = 2^{26.35-22.78} = 11.88$$

Similarly, the ratio of Histone 2B mRNA present in *A. nidulans* 3162 compared to *A. nidulans* 3168 is found by

$$R_H = 2^{C_{HT2}-C_{HT1}} = 2^{18.57-18.13} = 1.357$$

Correcting for the amount of sample loaded to find the relative expression of f-GnTI at the mRNA level,

$$R = \frac{R_E}{R_H} = 8.754$$

Therefore, *A. nidulans* 3162 expresses f-GnTI at the mRNA level 8.754 times more than *A. nidulans* 3168.

4.3. Western Blot

A Western blot was performed to verify expression on f-GnTI at the protein level in *A. nidulans* 3162 and 3168. Protein was prepared from lysate of *A. nidulans* 2162, 3162, and 3168. The concentration was measured using a BCA assay and run in duplicate on an SDS-PAGE gel, loading twice the sample volume in the second lane for each sample compared to the first. Actin was blotted for as a loading control.

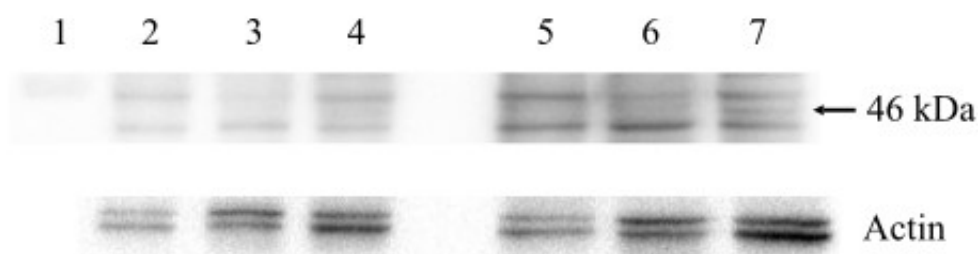


Figure 5. Western blot using anti-GnTI primary antibody. Lane 1) Protein standard, showing a 50 kDa band. 2) 8 µg *A. nidulans* 2161 protein from lysate. 3) 8 µg *A. nidulans* 3162 protein from lysate. 4) 8 µg *A. nidulans* 3168 protein from lysate. 5) 16 µg *A. nidulans* 2161 protein from lysate. 6) 16 µg *A. nidulans* 3162 protein from lysate. 7) 16 µg *A. nidulans* 3168 protein from lysate.

GnTI is expected to be seen with a molecular weight of 46 kDa. A band at 46 kDa can be seen in lane 7, containing 16 µg of protein from *A. nidulans* 3168 lysate; however, this band is not visible in any of the other lanes.

4.4. Lectin Blot

Protein was prepared from cell lysate and run on an SDS-PAGE gel. Additionally, spent media from the strains, which should contain secreted protein, was also run on the gel. The gel was then used to prepare a lectin blot using WGA, which has

binding specificity for GlcNAc and sialic acid, thus making any proteins with terminally GlcNAcylated or sialylated glycans visible.

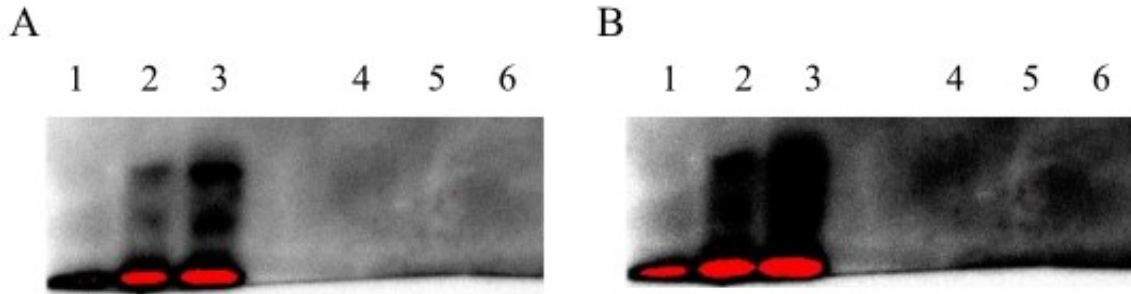


Figure 6. Lectin blot using WGA. Lane 1) 6 μ g protein from *A. nidulans* 2161 lysate. 2) 6 μ g protein from *A. nidulans* 3162 lysate. 3) 6 μ g protein from *A. nidulans* 3168 lysate. 4) 6 μ g protein from *A. nidulans* 2161 spent media. 5) 6 μ g protein from *A. nidulans* 3162 spent media. 6) 6 μ g protein from *A. nidulans* 3168 spent media. A) 40 second camera exposure time. B) 60 second camera exposure time. The red spots are a result of overexposure.

The lectin blot shows binding to many proteins from *A. nidulans* 3162 and 3168 lysate, but few in *A. nidulans* 2161 lysate and none in the media of any of the tested strains. To verify the presence of proteins in the media, the samples from the lectin blot above were run in an additional SDS-PAGE gel, which was then stained using Coomassie Blue. Coomassie Blue stains all proteins, making any proteins present in the gel visible.

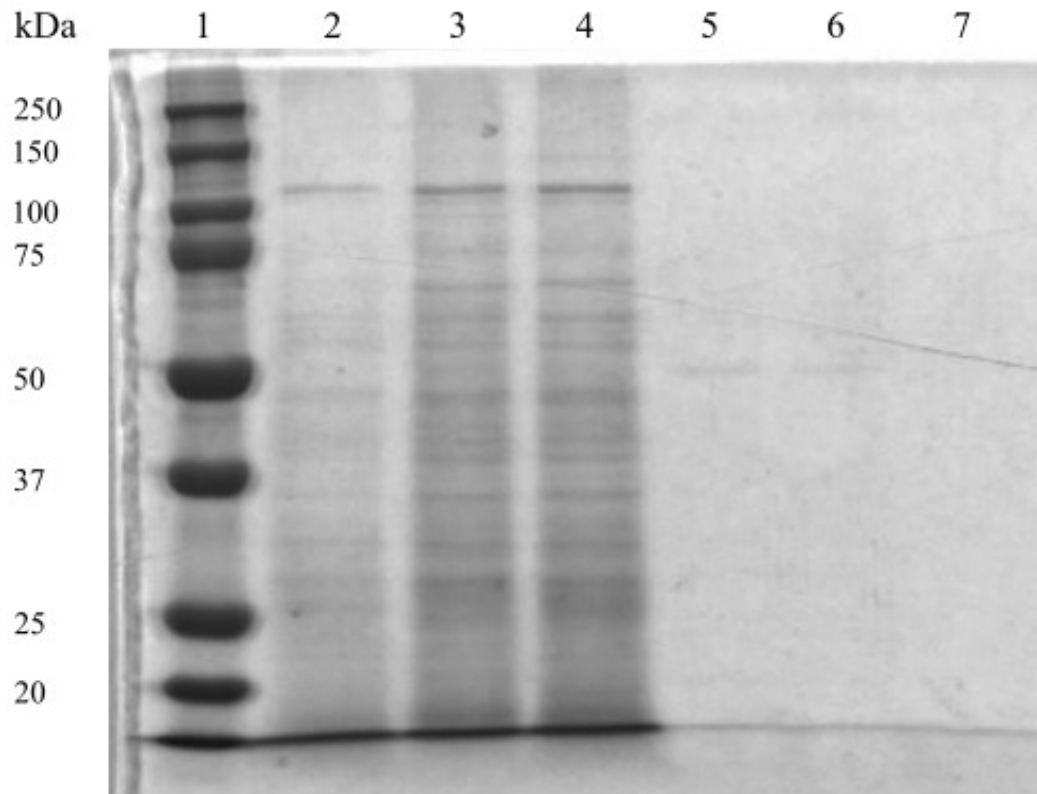


Figure 7: Coomassie Blue stain of protein extracts. Lane 1) Protein standard. 2) 10 µg protein from *A. nidulans* 2161 lysate. 3) 10 µg protein from *A. nidulans* 3162 lysate. 4) 10 µg protein from *A. nidulans* 3168 lysate. 5) 10 µg protein from *A. nidulans* 2161 spent media. 6) 10 µg protein from *A. nidulans* 3162 spent media. 7) 10 µg protein from *A. nidulans* 3168 spent media.

It can be seen in Figure 7 that proteins are present in the samples derived from cell lysate, which is consistent with the Western and lectin blots. While some faint bands can be seen in lanes 5 and 6 of the Coomassie Blue-stained gel, proteins are largely absent.

Chapter 5. Discussion

5.1. Insertion of GnTIIcas

Sequencing of PCR products showed the presence of GnTI when using primers och1_US_F and och1_DS_R shows the presence of GnTI, however, sequencing using och1_US800_F and och1_DS800_R showed the presence of och1, with no alignment for GnTI. This is because och1_US_F and och1_DS_R will anneal to the ends of the GnTIIcas insert regardless of its location in the genome. Conversely, och1_US800_F and och1_DS800_R are specific for the genomic location of och1 since they anneal to regions upstream and downstream of och1, respectively, but not to GnTIIcas itself. Sequencing using these primers would return a sequence which aligns with och1 should GnTIIcas not have been successfully inserted into och1's location in the genome. Taken together, these results indicate that in *A. nidulans* 3162 and 3168, GnTIIcas has been inserted, however not to the target site of the och1 gene. As a result, the native och1 gene is still present in these strains. Rather than have been targetedly inserted, GnTIIcas was randomly inserted into the genomes of the transformant strains. This most likely occurred due to the length of the upstream and downstream regions flanking GnTI-dTM. Targeted gene insertion is usually accomplished in *A. nidulans* using at least 800 bp of homologous region each upstream and downstream of the insert [19]. Longer lengths of homologous regions have been shown to result in higher transformation efficiency in *A. nidulans*. In this study, only about 400 bp of homologous region was used for each of the upstream and downstream regions, which was not enough to result in targeted insertion of GnTIIcas. Nonetheless, GnTIIcas is present in the genomes of *A. nidulans* 3162 and 3168.

5.2. mRNA Expression of f-GnTI

The results of the qRT-PCR show that *A. nidulans* 3162 expresses f-GnTI close to nine times more than *A. nidulans* 3168 at the mRNA level, while *A. nidulans* 2161 showed no expression of f-GnTI or any off-target amplicons. Furthermore, expression of histone 2B mRNA is shown in all samples, confirming that cDNA is present and intact in all samples. This indicates that f-GnTI is successfully being expressed in *A. nidulans* 3162 and 3168 and is a result of the transformation with GnTIIcas. The difference in f-GnTI expression levels between the two transformant strains is because the random insertion of GnTIIcas resulted in the gene integrating into a different location in the genome in each of these two strains. There are many ways in which genomic location can affect the expression levels of a gene. Firstly, due to the way genomic DNA is folded in the cell, some locations of the genome are more accessible by transcription machinery than others. The location at which GnTIIcas was integrated into *A. nidulans* 3162 may be more readily accessible than where it was integrated into *A. nidulans* 3168. Additionally, the genomic location may have stronger promoters and/or enhancers in *A. nidulans* 3162 than in *A. nidulans* 3168, which would also result in higher mRNA expression levels.

5.3. Protein Expression and Activity

Protein expression levels were first assessed by Western blot. The Western blot showed multiple bands, indicating that one of the antibodies being used has some non-specific binding. Any bands that are the result of non-specific binding should be present in all samples, as their expressed proteome is expected to be the same except for the presence of f-GnTI. Amidst these excess bands, there is a band at 46 kDa which appears

only in the protein from *A. nidulans* 3168 lysate. 46 kDa is the expected molecular weight for GnTI, implying that f-GnTI is expressed at the protein level the most or only in *A. nidulans* 3168. Interestingly, this is in contrast to what would be expected based on the difference in mRNA expression between the transformant strains as indicated by the qRT-PCR. There are many complexities in post-transcriptional modification of mRNA and translation, however, so the lack of correlation between mRNA expression levels and protein expression is not abnormal.

The lectin blot appears significantly darker in lanes loaded with protein from the transformant strains' lysates compared to lanes with protein from *A. nidulans* 2161 lysate. This indicates that there is significantly more terminal GlcNAcs on the glycans of the transformant strains compared to the parent strain, signifying that f-GnTI is active in these two strains. All three of the lanes containing protein samples from lysate have a blot at the bottom of the membrane. This could be glycans that were incomplete, as GlcNAcs are exposed on fungal N-glycans before the first mannoses are added while the glycan is still attached to the dolichol-pyrophosphate on the ER surface (Figure 1). The blot pertaining to *A. nidulans* 3168 lysate is noticeably darker than that pertaining to *A. nidulans* 3162 lysate, implying that f-GnTI is more active in *A. nidulans* 3168 than it is in *A. nidulans* 3162. This is consistent with the results of the Western blot, which show a higher protein concentration in *A. nidulans* 3168. This also implies that f-GnTI may be present on the Western blot in the *A. nidulans* 3162 samples, but at too low a concentration to be seen. The lectin did not bind to any glycoproteins in the spent media.

To investigate whether there were any proteins in these samples at all, an additional gel was run using the same samples and stained using Coomassie Blue, which

would reveal any proteins present. Coomassie Blue staining did not show a significant amount of protein in the spent media, indicating that the lack of lectin binding was a result of a lack of protein in the samples. Since the sample volumes loaded were based on protein concentration as determined by a BCA assay, it may be that the media composition used induces false positives in this assay. BCA being a colorimetric assay, it is easy to imagine some component of the minimal media absorbs light at a similar wavelength as the assay reagents. To further support this hypothesis, samples containing high amounts of protein visibly turn purple during a BCA assay. Furthermore, one of the components of the minimal media used in this experiment, Hutner's Solution, is a dark purple color. Therefore, it is possible that Hutner's Solution has a similar absorbance wavelength as the BCA reagent and thus interferes with the BCA assay.

The lane pertaining to *A. nidulans* 2161 cell lysate in the Coomassie Blue gel is noticeably lighter than the lanes pertaining to *A. nidulans* 3162 and 3168 lysates. This indicates that less protein was loaded into that lane. While this implies that less protein was also loaded into the corresponding lane for the lectin blot, the difference darkness of the lanes in the Coomassie Blue gel, and by extension the protein concentration, is not enough to account for the vast difference in darkness of the blots on the lectin blot. Furthermore, the additional exposure time causes the streaks in lanes 2 and 3 of the lectin blot to become significantly darker, whereas lane 1 does not appear to change much. If GlcNAcylated glycans were present in a significant concentration, the extra exposure time should have caused the lane to become darker. Therefore, sample from *A. nidulans* 2161 is not simply lighter due to a lower concentration of protein having been added, but rather that there is not a significant amount of GlcNAcylated glycans.

The lectin used to produce this lectin blot was WGA, which has binding specificity for sialic acid in addition to GlcNAc [27]. While this would raise concerns in some systems, fungi are not known to naturally produce sialic acid and no gene pertaining to sialic acid was introduced, therefore, it is highly unlikely that the WGA binding observed in the lectin blot is a result of binding to sialic acid [11, 28].

Chapter 6. Conclusion

The primary aim of this study was to produce an *Aspergillus nidulans* strain which could produce an N-glycan containing one GlcNAc, which could then serve as a foundation for further humanization of the glycosylation pathway. While GnTIIcas was not successfully targetedly inserted into och1's location in the genome, the resulting random insertion still provided sufficient expression of f-GnTI to cause a change in the transformant strains' glycans, presumably being the addition of terminal GlcNAcs. Continuing forward, it may be necessary to delete och1 to further reduce unwanted mannosylation of N-glycans.

Furthermore, the success of the och1-GnTI fusion protein in affecting glycosylation is indicative of a simpler method for glycoengineering going forward. Research groups have formerly used combinatorial libraries for similar studies, which involves creating libraries of random fusions between several gene and signal peptide combinations and high throughput transformant screening. Comparatively, this strategy of selecting signal peptides and transmembrane domains from fungal glycosylation genes to fuse with mammalian glycosylation genes is relatively simple and may continue to be useful in future glycoengineering endeavors.

Chapter 7. Future Work

This study has been the beginning of a long process which is required to fully humanize the fungal glycan. As we were unable to perform any structural studies of the N-glycans resulting from this work, it would be useful to perform such studies to elucidate the specific manner in which the insertion of f-GnTI affected the glycosylation. Following this work, och1 can be deleted to reduce mannosylation of N-glycans, as well as other fungal mannosyltransferases such as mnn9 and van1 which function in the fungal Golgi apparatus. Additionally, GnTII needs to be inserted into the strain. Not only would this function to add GlcNAc to the α -1,6 mannose branch of the glycans, but it could also serve to reduce mannosylation of glycans by way of competing for substrate with mannosyltransferases, as has been seen in the yeast *Pichia pastoris* [29]. After the addition of GnTII, galactosylation and sialylation of the N-glycans need to be achieved. Once sialylation of glycans has been achieved in *A. nidulans*, a functional therapeutic glycoprotein product could then be introduced and tested for efficacy.

Aspergillus nidulans serves as a model organism for filamentous fungi due to the fact that it is relatively easy to work with and well understood; however, *Aspergillus niger* is known to secrete higher amounts of protein, and therefore is more industrially relevant. Once the methodologies for humanizing the glycan in *A. nidulans* have been determined, it would still be more beneficial from an industrial perspective to transfer the genetic technology to *A. niger*. After humanization of glycosylation in fungi has been achieved, it would allow for cheaper production of pharmaceutical glycoproteins.

References

1. Jacobs, P. P., Geysens, S., Vervecken, W., Contreras, R., & Callewaert, N. (2008). Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nature protocols*, 4(1), 58.
2. Varki, A., & Lowe, J. B. (2009). Biological roles of glycans. In *Essentials of Glycobiology. 2nd edition*. Cold Spring Harbor Laboratory Press.
3. Betenbaugh, M. J., Tomiya, N., Narang, S., Hsu, J. T., & Lee, Y. C. (2004). Biosynthesis of human-type N-glycans in heterologous systems. *Current opinion in structural biology*, 14(5), 601-606.
4. Hamilton, S. R., Bobrowicz, P., Bobrowicz, B., Davidson, R. C., Li, H., Mitchell, T., ... & Wildt, S. (2003). Production of complex human glycoproteins in yeast. *Science*, 301(5637), 1244-1246.
5. Hamilton, S. R., Davidson, R. C., Sethuraman, N., Nett, J. H., Jiang, Y., Rios, S., ... & Hopkins, D. (2006). Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science*, 313(5792), 1441-1443.
6. Anyaogu, D. C., & Mortensen, U. H. (2015). Manipulating the glycosylation pathway in bacterial and lower eukaryotes for production of therapeutic proteins. *Current opinion in biotechnology*, 36, 122-128.
7. Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature biotechnology*, 22(11), 1393.
8. Maras, M., van Die, I., Contreras, R., & van den Hondel, C. A. (1999). Filamentous fungi as production organisms for glycoproteins of bio-medical interest. In *Glycotechnology* (pp. 19-27). Springer, Boston, MA.
9. Hamilton, S. R., & Gerngross, T. U. (2007). Glycosylation engineering in yeast: the advent of fully humanized yeast. *Current opinion in biotechnology*, 18(5), 387-392.
10. Chung, C. Y., Majewska, N. I., Wang, Q., Paul, J. T., & Betenbaugh, M. J. (2017). SnapShot: N-glycosylation processing pathways across kingdoms. *Cell*, 171(1), 258-258.
11. Deshpande, N., Wilkins, M. R., Packer, N., & Nevalainen, H. (2008). Protein glycosylation pathways in filamentous fungi. *Glycobiology*, 18(8), 626-637.
12. Donadio-Andréi, S., Iss, C., El Maï, N., Calabro, V., & Ronin, C. (2012). Glycoengineering of protein-based therapeutics. *Carbohydr Chem*, 38, 94-123.
13. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., & Ashwell, G. (1971). The role of sialic acid in determining the survival of glycoproteins in the circulation. *Journal of Biological Chemistry*, 246(5), 1461-1467.
14. Park, E. I., Manzella, S. M., & Baenziger, J. U. (2003). Rapid clearance of sialylated glycoproteins by the asialoglycoprotein receptor. *Journal of Biological Chemistry*, 278(7), 4597-4602.

15. Armstrong, J. K., Hempel, G., Koling, S., Chan, L. S., Fisher, T., Meiselman, H. J., & Garratty, G. (2007). Antibody against poly (ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*, 110(1), 103-111.
16. Anyaogu, D. C., Hansen, A. H., Hoof, J. B., Nielsen, K. F., Hobley, T. J., Yang, S., ... & Mortensen, U. H. (2016). Eliminating N-linked high-mannose glycans in *Aspergillus nidulans* by glycoengineering. Unpublished manuscript, Technical University of Denmark, Lyngby, Denmark.
17. Eades, C. J., & Hintz, W. E. (2000). Characterization of the Class I α -mannosidase gene family in the filamentous fungus *Aspergillus nidulans*. *Gene*, 255(1), 25-34.
18. Nielsen, J. B., Nielsen, M. L., & Mortensen, U. H. (2008). Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. *Fungal Genetics and Biology*, 45(3), 165-170.
19. Nayak, T., Szewczyk, E., Oakley, C. E., Osmani, A., Ukil, L., Murray, S. L., ... & Oakley, B. R. (2005). A versatile and efficient gene targeting system for *Aspergillus nidulans*. *Genetics*.
20. Choi, B. K., Bobrowicz, P., Davidson, R. C., Hamilton, S. R., Kung, D. H., Li, H., ... & Gerngross, T. U. (2003). Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. *Proceedings of the National Academy of Sciences*, 100(9), 5022-5027.
21. Nett, J. H., Stadheim, T. A., Li, H., Bobrowicz, P., Hamilton, S. R., Davidson, R. C., ... & Wildt, S. (2011). A combinatorial genetic library approach to target heterologous glycosylation enzymes to the endoplasmic reticulum or the Golgi apparatus of *Pichia pastoris*. *Yeast*, 28(3), 237-252.
22. Sun, X., Zhu, J., Bao, L., Hu, C., Jin, C., Harris, S. D., ... & Li, S. (2013). PyrG is required for maintaining stable cellular uracil level and normal sporulation pattern under excess uracil stress in *Aspergillus nidulans*. *Science China Life Sciences*, 56(5), 467-475.
23. Segato, F., Damásio, A. R., Gonçalves, T. A., de Lucas, R. C., Squina, F. M., Decker, S. R., & Prade, R. A. (2012). High-yield secretion of multiple client proteins in *Aspergillus*. *Enzyme and microbial technology*, 51(2), 100-106.
24. Nielsen, M. L., De Jongh, W. A., Meijer, S. L., Nielsen, J., & Mortensen, U. H. (2007). Transient marker system for iterative gene targeting of a prototrophic fungus. *Applied and environmental microbiology*, 73(22), 7240-7245.
25. Boppidi, K. R., Ribeiro, L. F. C., Iambamrung, S., Nelson, S. M., Wang, Y., Momany, M., ... & Marten, M. R. (2018). Altered secretion patterns and cell wall organization caused by loss of PodB function in the filamentous fungus *Aspergillus nidulans*. *Scientific reports*, 8(1), 11433.

26. Nødvig, C. S., Nielsen, J. B., Kogle, M. E., & Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One*, *10*(7), e0133085.
27. Immel, F., Broussard, C., Catherinet, B., Plasseraud, L., Alcaraz, G., Bundeleva, I., & Marin, F. (2016). The shell of the invasive bivalve species *Dreissena polymorpha*: biochemical, elemental and textural investigations. *PloS one*, *11*(5), e0154264.
28. Maras, M., van Die, I., Contreras, R., & van den Hondel, C. A. (1999). Filamentous fungi as production organisms for glycoproteins of bio-medical interest. In *Glycotechnology* (pp. 19-27). Springer, Boston, MA.
29. Bobrowicz, P., Davidson, R. C., Li, H., Potgieter, T. I., Nett, J. H., Hamilton, S. R., ... & Rausch, S. (2004). Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose. *Glycobiology*, *14*(9), 757-766.

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Graduate Researcher – Johns Hopkins University, Betenbaugh Lab (August 2016 – October 2018)

- Successfully expressed human membrane protein in *Aspergillus nidulans* to humanize glycosylation, laying the groundwork for future projects and establishing a simple and effective method for expressing glycosylation genes in the fungal Golgi apparatus.
- Designed and ran PCR, RT-qPCR, Western, and lectin blot experiments to verify successful genetic engineering, showing expression and activity of transgenic enzyme.
- Removed selection marker from engineered strains to allow for subsequent genetic engineering.
- Optimized transformation protocol for use with the glycoengineered strain.
- Established a collaboration with a lab from another university which resulted in NSF funding for a new project.
- Coordinated and ran experiments at two different universities.
- Trained newer students on lab techniques.
- Ordered supplies as needed.

Associate Scientist – Abbott Molecular (August 2015 – August 2016)

- Prepared samples and conducted experiments according to FDA- and CE-approved protocols to validate multiplex PCR-based diagnostics system.
- Utilized automatic liquid handling devices for magnetic DNA extraction and desalting of samples.
- Used MALDI-TOF mass spectrometry of amplified DNA to characterize pathogenic contents of original sample.
- Utilized bioanalyzer to screen for antibiotic resistance genes.
- Registered samples in company database using FDA and CE protocol sample naming guidelines and resolved sample name discrepancies.
- Assisted in writing of FDA protocol.
- Trained colleagues on lab protocols.
- Maintained laboratory supply stocks and requested supplies as needed.

Lab Assistant – Moores Cancer Center, UCSD, Reid Lab (July 2014 – June 2015)

- Maintained HEK 293 cell lines used in development of viral vectors for the purpose of melanoma treatment.
- Routinely measured mouse tumors to monitor tumor growth.
- Purified viral DNA.
- Monitored laboratory glassware and consumables and autoclaved equipment as needed.
- Inventoried supply stocks.

Laboratory Skills

- | | |
|--|--|
| • Transgenic expression in filamentous fungi | • Western blot |
| • Fusion PCR | • Lectin blot |
| • qPCR | • His-tag affinity protein purification |
| • RTPCR | • MALDI-TOF mass spectrometry |
| • DNA and RNA purification | • Selection marker recycling |
| • Mouse handling | • Filamentous fungal, yeast, adherent mammalian, and bacterial cell cultures |

Software Skills

- | | |
|-------------------------------------|---------------------|
| • Microsoft Word, PowerPoint, Excel | • Adobe Illustrator |
| • NCBI BLAST | • Maple |

Publications

- Chung, C. Y., Majewska, N. I., Wang, Q., Paul, J. T., & Betenbaugh, M. J. (2017). SnapShot: N-glycosylation processing pathways across kingdoms. *Cell*, 171(1), 258-258.